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Functional Roles of Human Kallikrein-related Peptidases*

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Kallikrein-related peptidases constitute a single family of 15 (chymo)trypsin-like proteases (KLK1–15) with pleiotropic physiological roles. Aberrant regulation of KLKs has been associated with diverse diseases such as hypertension, renal dysfunction, skin disorders, inflammation, neurodegeneration, and cancer. Recent studies suggested that coordinated activation and regulation of KLK activity are achieved via a complex network of interactions referred to as the "KLK activome." However, it remains to be validated whether these hypothetical KLK activation cascade pathways are operative *in vivo*. In addition, KLKs have emerged as versatile signaling molecules. In summary, KLKs represent attractive biomarkers for clinical applications and potential therapeutic targets for common human pathologies.

The KLK Family

Kallikrein-related peptidases (known as KLKs) (1) constitute a single family of 15 highly conserved trypsin- or chymotrypsin-like serine proteases encoded by the largest uninterrupted cluster of protease-encoding genes (*KLK1–15*) in the human genome. KLK3 or PSA[2] is the most widely known KLK due to its application in diagnosis and monitoring of prostate cancer (2, 3). *KLK* genes share multiple similar structural features, including exon/intron organization, conserved intronic intervals, and exon lengths (2). All *KLK* genes encode single-chain preproenzymes with lengths varying between 244 and 293 amino acid residues and ~40% protein identity among each other. Multiple *KLK* genes are often coexpressed in normal tissues, and they are usually coordinately deregulated in disease states, pointing to common mechanisms of regulation. Indeed, the expression of most *KLK* genes is regulated by nuclear receptor signaling, whereas KLK zymogen activation is thought to proceed via complex proteolytic cascades that lead to sequential activation of multiple KLK enzymes that, in turn, regulate important normal and pathobiological processes such as semen liquefaction, skin desquamation, innate immunity, neurodegeneration, degradation, and remodeling of ECM. In addition to KLK3/PSA, certain KLKs are aberrantly expressed in different types of cancer and provide novel tumor markers (mRNA, protein, genomic DNA methylation) for cancer diagnosis, prognosis, and monitoring (4). In addition, KLK6 has been suggested as a potential marker for Alzheimer disease (2).

Because of their significant roles in common human pathologies, KLKs are currently under study as potential therapeutic targets. Prostate-specific expression of KLK3/PSA has been exploited for PSA-targeting therapeutic strategies that include PSA-loaded antigen-presenting cells and PSA vaccines for prostate cancer (reviewed in Ref. 5). In addition, PSA-activated prodrugs have been designed for treatment of prostate cancer based on the fact that serum PSA is mostly enzymatically inactive, whereas in the prostate gland, it is found in its active form (6). Notably, administration of recombinant KLK6 to mice with EAE resulted in the production of anti-KLK6 antibodies that inhibited its enzymatic activity, attenuated the severity of symptoms, and delayed the course of disease progression (7). Finally, a synthetic KLK1 inhibitor was shown to suppress breast cancer cell invasiveness, suggesting that KLK activity could be targeted for anticancer therapies (8).

Regulation of KLKs: The KLK Activome

Regulation of KLK activity occurs at multiple levels that involve genomic aberrations (mutations, gene amplifications, or rearrangements) and transcriptional, post-transcriptional, and/or post-translational mechanisms. More specifically, it was shown that multiple *KLK* genes exhibit copy number variations in ovarian tumors (9). Usage of alternative promoters was described for the synthesis of multiple transcripts by the *KLK6* (10) and *KLK11* (11) genes. Based on extended variations in their 5′-untranslated regions, additional *KLK* genes may also be transcribed via multiple promoters. Single-nucleotide polymorphisms correlated with differential *KLK* expression levels, whereas *KLK3*/*PSA* was identified as a candidate susceptibility gene for prostate cancer (12). In addition, it is well established that transcription of *KLK* genes in various tissues is under the control of steroid hormones (2) and vitamin D receptor signaling (13, 14). In addition, we and others (2, 13) have shown that DNA methylation and possibly other epigenetic mechanisms lead to silencing of certain *KLK* genes in cancer cells. KLK activity is further regulated by the production of multiple alternatively spliced transcript variants that mostly encode inactive KLK isoforms (15).

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[2] The abbreviations used are: PSA, prostate-specific antigen; ECM, extracellular matrix; EAE, experimental autoimmune encephalomyelitis; MMP, matrix metallopeptidase; uPA, urokinase-type plasminogen activator; PAR, protease-activated receptor; NS, Netherton syndrome; IGF, insulin-like growth factor; TGFβ, transforming growth factor β; SG, semenogelin.

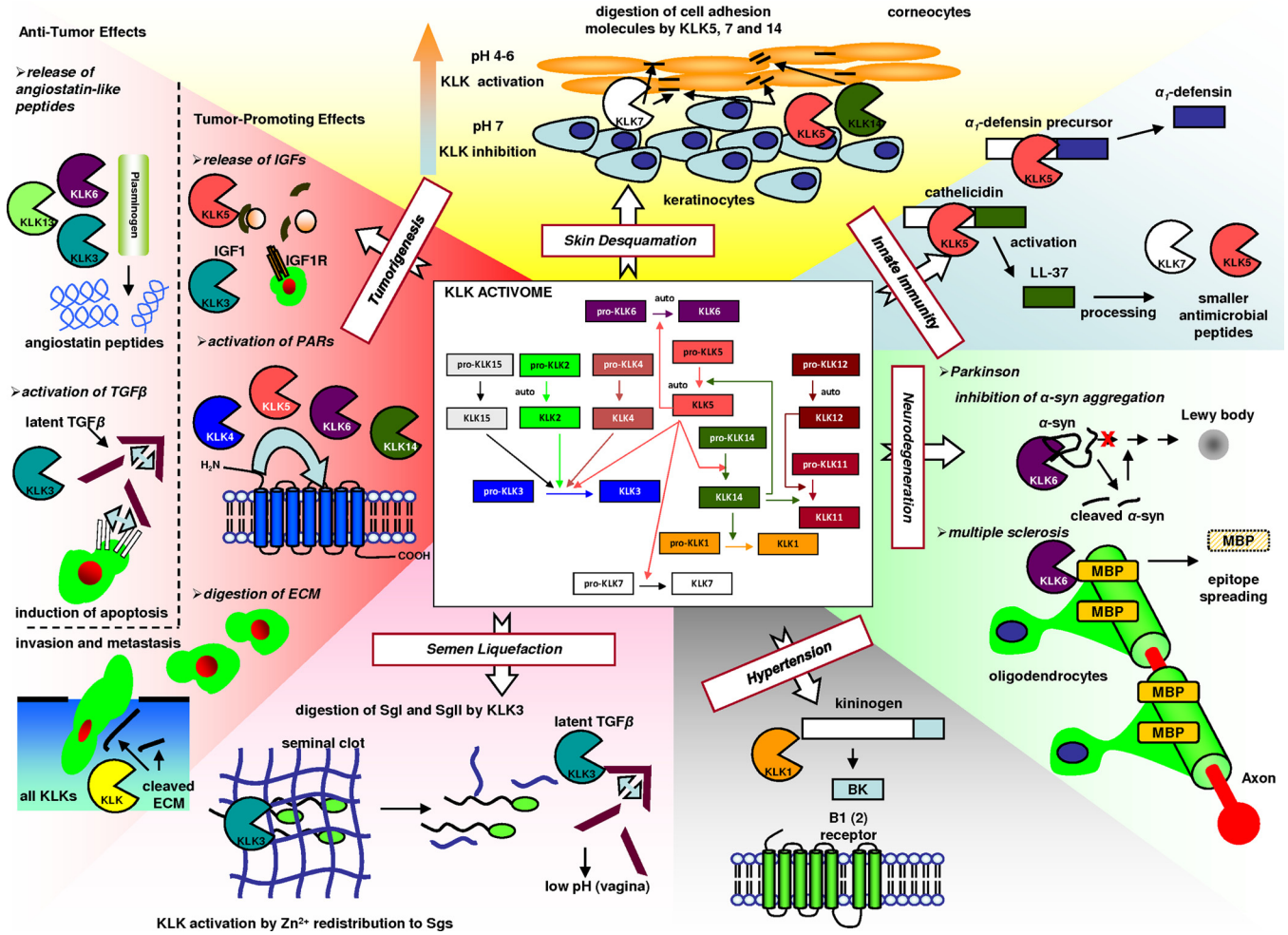

FIGURE 1. **Regulatory KLK cascades in normal physiology and disease states.** Proteolytic activities produced by zymogen activation via the KLK activome are involved in processes of skin desquamation, innate immunity, hypertension, semen liquefaction, neurodegeneration, and tumor-promoting or -inhibiting effects. Notably, certain KLKs exert pleiotropic functions by activating molecules involved in multiple processes, *e.g.* cathelicidin, which is involved in skin desquamation and innate immunity. It should be noted that only those KLKs that have been shown to activate recombinant pro-KLKs were included in the depicted KLK activome. *IGF1R*, IGF 1 receptor; *a-syn*, α-synuclein; *MBP*, myelin basic protein; *BK*, bradykinin; *B1*, bradykinin-1.

KLK proteins are synthesized as inactive prepro-forms that are proteolytically processed to secreted inactive pro-forms. Subsequently, pro-KLKs are activated to mature peptidases by specific proteolytic removal of their N-terminal propeptide either via autocatalytic activity or by another KLK or other endopeptidases. The term "KLK activome" was introduced to describe the serial activation of KLK zymogens by other mature KLKs (16). Our present understanding of activation profiles and the completed KLK activome is based on *in vitro* proteolytic cleavage of KLK propeptides and activation of recombinant pro-KLK proteins, and it is hypothesized to involve a complex network of activation events (autolytic, reciprocal cross-activations, reverse activations), as depicted in Fig. 1.

Following activation, mature KLK enzymes are amenable to inactivation by endogenous inhibitors such as, for example, the kallistatin, which is a specific inhibitor of KLK1, and LEKTI (lymphoepithelial Kazal-type inhibitor), which is encoded by *SPINK5* (serine protease inhibitor Kazal-type 5). LEKTI is a secreted serpin that requires proteolytic cleavage for generation of bioactive fragments that act as specific inhibitors of serine proteases, including certain KLKs (17). Inhibition of KLK activity by serine protease inhibitors (serpins) occurs through an irreversible suicide substrate mechanism that is referred to as the "inhibitory pathway." KLK-inhibitor complexes such as KLK3-α_2-macroglobulin and KLK3-α_1-antichymotrypsin have been identified *in vivo* (2). In addition, autocatalytic inactivation via internal cleavage has been shown for KLK6 and other KLKs (2), whereas inactivation may also occur through internal cleavage by other proteases as demonstrated *in vitro* for the deactivation of KLK11 by plasmin (18). An interesting feature of these enzymes is that activation of some KLK zymogens by another KLK is followed by subsequent internal cleavage and inactivation by the same or different mature KLK, as was demonstrated for the serial activation of pro-KLK3/PSA by KLK5 and its subsequent inactivation upon prolonged incubation (19). Zn^{2+} and pH are also very important reversible inhibitors of KLK enzymatic activities and are considered important regulators of KLK functions, as discussed below.

Structure and Activity

A number of recent studies described the resolution of crystal structures for KLK1 (20), KLK3 (21), KLK4, -5, and -7

(reviewed in Ref. 22), KLK6 (23), and pro-KLK6 (24), which will facilitate the detailed description of their substrate specificity. Common structural features of KLKs were revealed, including the two interacting β-barrels and α-helices bridged by the active site. Protein folding is facilitated by five or six disulfide bonds. Among KLKs, only KLK1, KLK2, and KLK3/PSA contain the characteristic kallikrein loop of 9–11 amino acid residues located prior to the active-site Asp, which confers specificity for kininogenase activity, namely the ability to release kinin from kininogen. Notably, KLK3/PSA is not able to cleave kininogen. Removal of the propeptide results in the formation of a salt bridge between the a-ammonium group of Ile/Leu^{16} and the carboxylate of the Asp^{194} side chain that is important for conformational rigidity of the active protease (22). An important characteristic of KLK3 is that two different conformations can be adapted by the 11-amino acid kallikrein loop, which can acquire either a closed or an open conformation that leads to either mature intact KLK3 with no enzymatic activity or enzymatically active KLK3, respectively. Conversion of the inactive (closed) to active (open) KLK3 conformation can be achieved by high salt concentration or by monoclonal antibodies that capture and stabilize active KLK3, as modeled recently (21).

As mentioned, each KLK contains a signal peptide (pre) of 16–30 amino acid residues that is cleaved prior to secretion, leaving the pro-KLK form. For KLK4, an isoform that lacks the prepeptide and localizes in the nucleus was identified in prostate cancer cells (25). Activation of pro-KLKs entails the removal of an N-terminal propeptide of 4–9 amino acids with the exception of KLK5, which carries a 37-amino acid long activation peptide. The cleavage site includes a P1 Arg or Lys, except for KLK4, which has Gln at P1. Accordingly, serine proteases with trypsin-like activity are required for activation of all other pro-KLKs, whereas MMP20 was suggested as the endogenous activator of KLK4 based on *in vitro* proteolysis data (26). The activity of KLKs is trypsin-like (KLK1, -2, -4–6, -8, -2, -13, and -15), chymotrypsin-like (KLK3, -7, and -9), or mixed-type (KLK11 and KLK14). Phage display, combinatorial libraries, and kinetic analyses were employed to characterize the substrate specificity of different KLKs. Paradoxically, KLK10/NES1 is considered to lack protease activity (16), which may be due to Ser substitution of Gly^{193} (chymotrypsin numbering) in KLK10/NES1. With very few exceptions, Gly^{193} in the oxyanion hole is highly conserved in serine proteases, and its role is the stabilization of the oxyanion intermediate during hydrolysis of the peptide bond. It should be noted, however, that using a library of small peptide substrates, Debela *et al.* (27) reported activity for KLK10 with ambivalent specificity. This controversy is presently not resolved due to the lack of the three-dimensional structure and of known physiological substrates and could be due to a very restricted specificity of the KLK10/NES1 tumor suppressor. However, it is possible that KLK10/NES1 and probably other members of the KLK family exert biological roles independently of their serine protease activity, as demonstrated for KLK3/PSA, which produces reactive oxygen radicals in prostate cancer cells independently of its proteolytic activity (28).

Regulatory Cascades and Functional Roles of KLKs

It is well established that KLK1 can cleave low molecular weight kininogen to release kinin, which mediates signaling by a number of downstream targets. In addition, KLK1 can cleave proinsulin, low density lipoprotein, prorenin, precursor of atrial natriuretic factor, and other factors (29). Recently, it was shown that KLKs, especially KLK1, exert a protective role against lupus and anti-glomerular basement membrane-specific antibody-induced nephritis in mice and humans (30). Furthermore, human systemic lupus erythematosus and spontaneous lupus nephritis were found to be associated with single-nucleotide polymorphisms located on *KLK1* and *KLK3* promoters (30). The observation that *Klk1*$^{-/-}$ mice showed reduced ability for renal Ca^{2+} reabsorption led to the hypothesis that KLK1 could be another physiological regulator of Ca^{2+} homeostasis (31). Interestingly, *Klk1*$^{-/-}$ mice have normal blood pressure, but they are characterized by cardiac and vascular abnormalities (32).

In vitro studies showed that KLK2, -4, and -12 are able to activate pro-uPA to plasmin, which activates the uPA-uPA receptor-MMP proteolytic pathways known to be involved in the degradation and remodeling of ECM. The direct cross-talk between KLKs and MMPs is also indicated by the *in vitro* activation of pro-MMP2 and pro-MMP9 by KLK1 (2) and of pro-KLK4 by MMP20 protease, which is important for amelogenesis, namely the formation of tooth enamel (26).

Recent studies *in vitro* and in mice implicate KLK6 in inflammation of the central nervous system and in multiple sclerosis. Consistently, KLK6 is abundantly expressed at sites of demyelination in the EAE mouse model of multiple sclerosis and in lesions detected in the brains of human patients (7). Efficient proteolytic cleavage of myelin basic protein *in vitro* supports a role of KLK6 in demyelination and/or remyelination (23). A potential role of KLK6 in the physiological degradation of α-synuclein and in the pathogenesis of Parkinson disease and other synucleinopathies was suggested based on findings with cultured cells showing that KLK6 degrades α-synuclein and co-localizes with pathological inclusions such as Lewy bodies and glial cytoplasmic inclusions (33). This was also shown by immunofluorescence visualization in sections of post-mortem human brains and by co-immunoprecipitation experiments using extracts of mouse brains. Involvement of KLK6 in synucleinopathies is further sustained by an *in vitro* study showing that KLK6 is localized in mitochondria, and upon cellular stress, it is released into the cytoplasm, where limited proteolysis of α-synuclein by KLK6 activity yields fragmented α-synucleins that inhibit polymerization by reducing the amount of monomer, thus preventing the formation of aggregates, a hallmark of these pathologies (33). On the other hand, *Klk8*$^{-/-}$ mice are predisposed to global seizures, pointing to antiepileptogenic activity of KLK8 (34). Importantly, *Klk8*$^{-/-}$ mice exhibit attenuated demyelination and oligodendrocyte death in the EAE model (35). In addition, the proteolytic activities of KLK3, -6, and -13 *in vitro* were shown to produce angiostatin-like peptides with known antiangiogenic activity by limited proteolysis of plasminogen at specific internal sites (2).

Accumulating evidence suggests that KLKs are activators of PARs, known members of the G-protein-coupled receptor superfamily that are activated by partial proteolytic cleavage of their extracellular domains (36). These data are corroborated by observations in a mouse model of NS, in which KLK5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression. Interestingly, uncontrolled KLK5 activity due to lack of KLK5 inhibition by LEKTI was shown to trigger a proinflammatory and proallergic microenvironment in NS epidermis independently of the environment and the adaptive immune system. This illustrates the crucial role of protease signaling in skin inflammation (37). Importantly, KLKs are known to cleave IGF-binding proteins, leading to increased availability of IGFs that bind and activate their corresponding receptors and that, in turn, can modulate cell survival, mitogenesis, and differentiation. In addition, KLK3 activates latent TGFβ by cleaving the TGFβ-binding protein (2).

The large number of *KLK* genes and their coordinated regulation and tissue coexpression patterns led to the hypothesis that the encoded proteins could participate in proteolytic cascades, currently postulated to be involved in semen liquefaction, skin desquamation, neurodegeneration, and tumor-promoting or -inhibiting effects (Fig. 1). Because KLK5 can activate itself as well as pro-KLK2, -3, -6, -7, -11, -12, and -14, KLK5 is considered the initiator of putative KLK cascades (19). As mentioned, evidence for the operation of KLK activation cascades comes mainly from *in vitro* proteolysis of recombinant pro-KLKs (2, 16, 19, 38). It should be noted that activation of KLK zymogens may also involve other proteases such as specific MMPs and uPA (2).

It is well established that KLK3/PSA is the physiological enzyme responsible for the resolution of the seminal clot by digestion of SgI and SgII. However, KLK3 is secreted from the prostate as an inactive zymogen that requires activation. Recently, it was demonstrated by *in vitro* proteolysis that KLKs secreted in prostatic fluid can participate in a hypothetical cascade that leads to activation of pro-KLK3. Because it was shown that KLK5 is able to autoactivate and also activate several other pro-KLKs, it was speculated that KLK5 could be the key molecule for the initiation of the postulated prostate cascade. Importantly, prostatic fluid contains significantly elevated concentrations of Zn^{2+} ($\sim$2 mM) that were shown to block the enzymatic activities of most KLKs *in vitro*. During ejaculation, the prostatic fluid mixes with epididymal fluid that contains spermatozoa and with seminal vesicle fluid that contains SgI and SgII. SgI and SgII, along with fibronectin, form the seminal clot that entraps spermatozoa. Redistribution of Zn^{2+} to SgI and SgII, which have high affinity for Zn^{2+}, is expected to activate the KLK cascade that will eventually lead to activation of KLK3/PSA and digestion of the seminal clot. In prostate cancer, reduced concentrations of Zn^{2+} were measured in prostate lumen due to the established down-regulation of zinc transporter proteins. Presumably, low levels of Zn^{2+} cause activation of KLKs in prostate tissue and loss of prostate tissue architecture due to KLK-mediated degradation of ECM (19, 39). Moreover, the prostatic KLK cascade may play significant role(s) in bone metastasis of prostate cancer. Interactions between tumor cells and bone cells (osteoblasts) are critical for the establishment of metastatic tumors associated with drug resistance and high mortality. In particular, KLK4 is considered to mediate bone metastasis because *in vitro* experiments demonstrated that the enzymatic activity of KLK4 is required for increased migration of prostate cancer cells against osteoblast-secreted factors (40). Also, KLK4 could promote prostate cancer metastasis by activating pro-KLK3 to mature KLK3/PSA and pro-uPA to uPA, which is associated with invasion due to extensive degradation of ECM (2).

In skin, KLK activities are regulated mainly by LEKTI in combination with changes in microenvironmental pH, as shown by *in vitro* studies (17) and in *Spink5*$^{-/-}$ mice, an established animal model of NS. NS is a severe form of ichthyosis (*e.g.* enhanced desquamation) caused by mutations in *SPINK5* and lack of LEKTI, resulting in increased proteolytic activities of KLK5 and KLK7 (41). It has been demonstrated *in vitro* that LEKTI binding and inactivation of KLKs are reversed by a decrease in pH to the range 4.5–5.5 (17). It is known that the upper skin layer (stratum corneum) maintains a pH in this range. On the other hand, KLK5, -7, and -14, along with LEKTI, are produced in the lower skin layer (stratum granulosum), where the pH is almost neutral (38). KLK5 displays enzymatic activity both at acidic pH (4.5–5.5), which is found in the stratum corneum due to the "acid mantle," and at the neutral pH of the stratum granulosum. It is assumed that KLK5 autoactivates in the stratum granulosum, but its activity is quenched by immediate binding of LEKTI fragments. Dissociation of the KLK5-LEKTI complexes and release of active KLK5 enzyme occur as it diffuses into the stratum corneum, which maintains an acidic pH. Then, KLK5 activates KLK7 and KLK14, whereas active KLK14 augments KLK5 activity in a feedback loop as shown *in vitro* using recombinant enzymes (17). Active KLK5, -7, and -14 can digest the corneocyte binding proteins desmoglein, desmocollin, and corneodesmosin, leading to skin desquamation (38), as depicted in Fig. 1.

Based on the fact that several KLKs (KLK5–8 and KLK10–13) are present in human cervicovaginal fluid at exceptionally high concentrations (0.5–3 mg/liter), it was hypothesized that KLK activities may participate in desquamation of vaginal epithelial cells, reminiscent of the skin desquamation process (42). In addition, KLKs could be involved in the proteolytic release and processing of antimicrobial peptides found in vaginal fluid. Indeed, it was shown *in vitro* that KLK5 can activate the α_1-defensin precursor to its mature form (42). Thus, KLK5 proteolytic activity at an epithelial interface may be linked to mechanisms underlying the regulation of innate immunity defense. This hypothesis is supported further by the fact that, in human skin, KLK5 and KLK7 were shown to control the activation of the human cathelicidin precursor protein CAP18 and also influence further processing to smaller peptides with increased antimicrobial activities (43). The importance of KLKs to antimicrobial activity *in vivo* is supported by the finding that epidermal extracts from *Spink5*$^{-/-}$ mice display significantly increased antimicrobial activity that was shown to be due to KLK-mediated processing of cathelicidin (43).

Recent functional studies show that aberrant regulation of KLKs interferes with different stages of cancer growth and pro-

gression, including tumor growth, dedifferentiation, angiogenesis, and metastasis. It was shown that KLK10/NES1 acts as a tumor suppressor in breast (44) and gastric (45) cancers. The mechanism(s) underlying the tumor suppressor function of KLK10/NES1 have not been described. Recently, it was shown that when KLK6 is expressed at physiological concentrations, it dramatically inhibits growth of primary breast tumors (46). Also, a number of studies implicate certain KLKs in epithelial-to-mesenchymal transitions, which represent a critical step in cancer metastasis. In prostate cancer cells, expression of KLK3 and KLK4 results in loss of E-cadherin and induction of expression of the mesenchymal marker vimentin, which represents a hallmark of epithelial-to-mesenchymal transitions (47). Nonetheless, in transgenic mice overexpressing KLK6, it was shown that KLK6 is implicated in E-cadherin shedding in epidermal keratinocytes (48), whereas KLK7 was shown to directly induce E-cadherin shedding *in vitro* (49). In contrast, re-expression of KLK6 results in marked reduction of vimentin expression in metastatic breast tumor cells (46). Overall, existing evidence points to dual roles of KLKs in cancer, as also described for other proteases (reviewed in Ref. 50). The function of KLKs may vary in different tissues, tumor types, and cancer stages and likely depends on the concentration and/or activity levels. In this respect, it was found that the tumor-protective effect(s) of KLK6 are restricted to normal concentrations of the protein, whereas marked overexpression of KLK6, also observed in a subset of breast tumors, seems to be associated with enhanced tumor growth (46). Tumor-associated constitutive overexpression of KLK6 was recently associated with hyperproliferation of cancer cells in non-small cell lung cancer. It was suggested that increased expression of KLK6 leads to accelerated cell cycles between the G_1 and S phases (51). Consistent with KLK roles as cell cycle regulators, KLK4 was reported as a proliferative factor when overexpressed in prostate cancer cells, where it was shown to affect the expression of cell cycle-related genes (25). Nonetheless, a recent study showed that in colon cancer cells, KLK6 is up-regulated via the K-Ras pathway, and this increased expression of KLK6 was correlated with enhanced migration and invasion of tumor cells (52). On the other hand, it was shown recently that KLK6 can evoke intracellular Ca^{2+} flux via PAR1 signaling in cultured neurons and via PAR1 and PAR2 in cultured astrocytes (53). However, these findings should be interpreted with caution because the levels of active KLK6 used in these experiments were orders of magnitude higher than those found physiologically. Cumulatively, recent advances revealed interesting and unexpected roles of KLKs; however, these are currently only partially characterized.

Conclusions and Outlook

KLKs represent a major proteolytic system operating in many tissues, but their biological roles are still not well defined. An increasing number of studies implicate aberrant regulation of KLKs in common human diseases and point to their clinical applicability as disease biomarkers but also as attractive targets for therapeutic intervention. In recent years, KLK serine proteases emerged as important players in the vast landscape of normal and disease-associated proteolysis. Several lines of data indicate that KLKs act individually and/or in complex networks or "KLK cascades" that may also involve cross-talk(s) with other serine or metalloproteases. It is currently known that KLKs activate signaling via the kallikrein-kinin system, PARs, and uPA and by processing of TGFβ and IGF-binding proteins. Most of these data were derived *in vitro*, and their significance *in vivo* awaits validation. Delineation of the complete KLK activome *in vivo* and identification of endogenous substrates for the KLK enzymes will allow for the detailed description of the versatile functional roles of KLKs in physiological and pathological processes. In addition, the identification of potent specific and selective inhibitors of KLKs mainly through high-throughput screening platforms and substrate-guided design (54) will aid the development of novel KLK activity-modulating agents and the discovery of presently unidentified pathways mediated by KLKs *in vivo*. In conclusion, the KLK field represents a largely unexploited area that will likely grow considerably over the next decade.

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